

Title

HISTONE CARBONYLATION OCCURS IN PROLIFERATING CELLS

Original article

García-Giménez José Luis^{1, 2, 4}, Velázquez Ledesma Ana María^{2, 4}, Esmoris Isabel^{1, 2, 4}, Romá-Mateo Carlos^{1, 3}, Sanz Pascual^{1, 3}, Viña José^{2, 4}, Pallardó Federico V^{1, 2, 4}.

¹ Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER) Valencia, Spain, ² Dept. Physiology. Medical School. Universitat de València. Valencia. Spain, ³ Instituto de Biomedicina de Valencia, CSIC. Valencia, Spain, ⁴ Fundación del Hospital Clínico Universitat de Valencia. FIHCUV-Incliva, Valencia, Spain.

Corresponding author

*Prof. Dr. Federico V. Pallardó
CIBERER. Biomedical Network Research Center for Rare Diseases
FIHCUV-INCLIVA
Departament de Fisiologia. Facultat de Medicina, Universitat de València
Avda. Blasco Ibañez N° 15.
E46010 - Valencia, Spain
Phone: +34963864646
FAX: +34963864642
E-mail: federico.v.pallardo@uv.es

Abstract

Chromatin is a dynamic structure formed mainly by DNA and histones and, chemical modifications on these elements regulate its compaction.

Histone post-translational modifications (PTMs) have a direct impact on chromatin conformation, controlling important cellular events like cell proliferation and differentiation. Redox related post-translational modifications may have important effects on chromatin structure and function offering a new intriguing area termed *Redox Epigenetics*.

Little is known about histone carbonylation, a PTM that may be related to modifications in the cellular redox environment. The aim of our study was to determine the carbonylation of the different histones during cell proliferation, a moment in cell life where important redox changes take place.

Here, we describe changes of histone carbonylation during cell proliferation in NIH3T3 fibroblasts. In addition, we have studied the variations of poly(ADP-ribosyl)ation and phospho-H2AX at the same time, because both modifications are related to DNA damage responses. High levels of carbonylation on specific histones (H1, H1⁰ and H3.1 dimers) were found when cells were in an active phase of DNA synthesis. The modification decreased when nuclear proteasome activity was activated. However, these results did not correlate completely with poly(ADP-ribosyl)ation and phospho-H2AX levels. Therefore, histone carbonylation may represent a specific event during cell proliferation.

We describe a new methodology named Oxy-2D-TAU Western blot that allowed us to separate and analyze the carbonylation pattern of the different histone variants. In addition we offer a new role for histone carbonylation and their implications in redox

epigenetics. Our results suggest that histone carbonylation is involved on histone detoxification during DNA synthesis.

Keywords:

Epigenetics, Histones, Carbonylation, poly(ADP-ribosyl)ation, cell proliferation

Introduction

Chromatin, a macromolecular complex composed of DNA and protein, is the heritable material of eukaryotic cells. The nucleosome is the repeating unit of the chromatin structure, in which DNA is wrapped around a core octamer unit composed by four histone proteins (H2A, H2B, H3 and H4 and/or their variants) assembled into the larger chromatin fiber including the linker histone H1. Histone linker H1 and its variant H1⁰ participate in specific mechanisms by which individual domains of these linker histones interact to facilitate chromatin condensation [1].

The histone proteins are subject to an extensive array of post-translational modifications (PTMs), including methylation, acetylation, ubiquitylation, SUMOylation, ADP-ribosylation, phosphorylation and citrullination [2-5] that contribute to codify epigenetic information, the ultimate regulating step in gene expression [6, 7].

One of the leading characteristics of cell nuclei is their capability to regulate their redox environment. It is assumed that the NAD⁺/NADH and NADP⁺/NADPH ratios are the same in the cytoplasm and the nucleus, thus there is no impediment for the diffusion of these molecules across the nuclear membrane [8, 9]. The concentration of free NADH

in the nucleus has been estimated to be 130 nM [9]. The NAD⁺ is the substrate of poly(ADP-ribose) polymerase 1 catalyzing poly(ADP-ribosyl)ation of target proteins [10].

In addition, glutathione (GSH) can detoxify free radicals, hydrogen peroxide and other peroxides, and regulate critical cellular and metabolic processes [11, 12]. GSH is found in a fairly high concentration of approximately 10 mM within cells and previous results obtained by our group demonstrate that GSH concentrates within the nucleus when most cells are proliferating [13]. However, GSH concentration decreases and redistributes uniformly between nucleus and cytoplasm when cells reach confluence [13].

Thus, the nucleus must be in a reduced state to facilitate proliferation and to protect DNA [14, 15]. Indeed redox dependent post-translational modifications of nuclear proteins [16], and specifically histones are critical for their function [17].

Aged-associated protein carbonylation has been studied extensively. But histone carbonylation has been reported in few studies [18, 19, 20]. Thus, to further increase the awareness of this poorly known post-translational modification on histones, we decided to study protein carbonylation not in a model of cell aging but during cell proliferation.

It has been suggested that carbonylation *in vivo* is the result, at least in part, of glycation reactions favored by ADP-ribose accumulation in the nucleus [18].

The (ADP-ribosyl)ation is a posttranslational modification of proteins that involves the addition of one or more ADP-ribose to different substrates [21, 22] and it constitutes an important redox epigenetic mark when it is produced on histones. These reactions are

involved in cell signaling and in the control of many cell processes, including DNA repair and apoptosis [23, 24]. The (ADP-ribosyl)ation is catalyzed by ADP-ribosyltransferase enzymes, which transfer the ADP-ribose group from nicotinamide adenine dinucleotide (NAD⁺) onto acceptors such as arginine, lysine, glutamic acid, or aspartic acid residues in proteins. The transfer of multiple ADP-ribose groups to proteins to form long branched chains is called poly(ADP-ribosyl)ation [25]. This protein modification is carried out by poly(ADP-ribose) polymerases (PARPs), which are found in most eukaryotes, but not in prokaryotes or yeast [26]. The poly(ADP-ribose) structure is involved in the regulation of several cellular events in the cell nucleus, like DNA repair and telomere maintenance and, PARP expression and activity have been linked with GSH levels during cell cycle [27]. In addition, PARPs recognize the Okazaki's fragments of the lagging strand of newly synthesized DNA as single strand breaks (SSBs) to mark the histones present on it [23-28].

In this work, we report the dynamic changes of carbonylation and poly(ADP-ribosyl)ation of histones during different times of cell culture and how histone carbonylation decreases after nuclear proteasome activation, indicating that histone carbonylation may be a redox epigenetic mark to prevent the fatal event of histone accumulation. In addition, we describe for the first time a new methodology that we have called Oxy-2D-TAU Western blot, and apply it to study carbonylation in different histone variants.

Material and methods

Cell culture, growing curves and cell treatments

The NIH3T3 cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, San Diego, CA, USA) and 1% penicillin/streptomycin (Invitrogen, San Diego, CA, USA) in 5% CO₂ in air at 37 °C in 75 cm² flasks.

Cells were treated with 1 µM of the proteasome inhibitor MG132 (Biomol, Enzo LifeScience, USA) during 4 h in order to ensure the inhibition of the proteasome avoiding collateral effects. The MG132 was added 24 and 48 h after seeding. For PARP inhibition experiments we used 3-aminobenzamide (3-ABA) (Sigma-Aldrich, USA). This drug was added at final concentration of 5 mM, 3 h after seeding and maintained during 24 and 48h of cell culture.

The growth curve was obtained by counting directly the cells using hemocytometer chamber at 6, 24, 48, 72 and 120 h after plating.

Measurement of DNA synthesis. Cell proliferation studies

Proliferation of cell lines was determined with the "Cell proliferation ELISA BrdU colorimetric kit" (Roche Diagnostics, Mannheim, Germany). Cells were cultured in a 96 wells plate at 6, 24, 48, 72, 120 h and processed according the manufacturer's protocol. The absorbance of the colorimetric final reaction was measured with the spectrophotometer spectra MAXPLUS 384 (Molecular Devices, Sunnyvale, CA, USA) at 370 nm, using the absorbance at 492 nm as reference wavelength, in three intervals of 5 min. The variation of absorbance is proportional to the incorporation of BrdU. This value was refereed to the total cell content determined by the sulforhodamine B assay,

which is used for cell density determination, based on the spectrophotometric measurement of cellular protein content [29].

Histone extraction protocol

Nuclei and histones were isolated as described earlier [30]. Cell nuclei were isolated by hypotonic lysis in buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, and 1 µL/mL of protease inhibitors (Roche Diagnostics, Mannheim, Germany). Pelleted nuclei were acid-extracted using 0.4 N sulfuric acid overnight 4 °C, precipitated with 99% trichloroacetic acid, washed with cold acetone, and resuspended in bi-distilled water.

Immunodetection of protein-bound carbonyl groups

To determine protein carbonyl groups, they were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by its reaction with 2,4-dinitrophenylhydrazine (DNPH), according to the procedure of Shacter *et al.* [31]. Briefly, 5 µL of proteins were denatured with 5 µL of 12% SDS. Then, 10 µL of 10 mM DNPH in 10% (v/v) trifluoroacetic acid were added to the protein solution. The reaction mixture was neutralized and prepared for SDS/PAGE by adding 7.5 µL of 2M Tris base containing 30% (v/v) glycerol. The derivatized samples were separated by electrophoresis in a 15% SDS-PAGE and transferred onto a nitrocellulose membrane.

Then, the membrane was blocked with 5% BSA in PBST for 1h, and incubated with anti-DNP antibody as described by the manufacturer of the Oxy-blot kit (OxyBlot Protein Oxidation Detection kit, Millipore Inc, Billerica, MA. USA).

Poly(ADP-ribosyl)ation studies

Poly(ADPriboseyl)ated proteins were studied by Western blotting, using 10 µg of nuclear proteins or histones. The electrophoresis was developed under reducing conditions. After electrophoresis, the proteins were transferred onto nitrocellulose membrane (Whatman Protran, Dassel, Germany). Membranes were blocked in 5% (w/v) non-fat dry milk in TBS-Tween for 1 h at room temperature, and probed against the anti-PAR antibody (Calbiochem, San Diego, CA, USA) at the dilution of 1:1000 in 1% (w/v) non-fat dry milk TBS-Tween overnight at 4°C, and secondary antibody, goat anti-mouse IgG (Calbiochem, San Diego, CA, USA) conjugated to horseradish peroxidase, at 1:7500 in 1% (w/v) non-fat dry milk for 1 h at room temperature. Detection procedure was performed using Amersham RPN 2106 ECL Western Blotting Detection Reagent (GE HealthcareBio-Sciences AB, Uppsala, Sweden). Images were captured in a Fujifilm LAS-600 and studied by ImageGauge 4.0.

2D-TAU Western blot to analyze histone poly(ADP-ribosyl)ation

2D-TAU gels were developed under two different conditions depending on the post-translational modification analyzed. For 2D-TAU WB analysis of poly(ADP-ribosyl)ation, typical 2D-TAU gel was run as described previously by Shechter *et al.* [29], with some modifications. Briefly, the histone extracts for each time were dried under vacuum and resuspended in loading buffer (6M urea, 0.02% (w/v) pyronin Y, 5% (v/v) acetic acid, 12.5 mg/ml protamine sulfate). First, samples were separated on TAU mini-gels (15% PAGE, 6 M urea, 5% acetic acid, 0.37% Triton X-100), 200V in running buffer (5% acetic acid) for 1h. The electrode switches were interchangeable in

order to produce the migration of histones to the cathode. When the first dimension was finished, lanes containing the samples were cut out, conditioned in 0.125 M Tris, pH 6.6 and the TAU gel slice was assembled on top of a 15% SDS-PAGE mini-gel. After the run, the gel was transferred onto a nitrocellulose membrane (Whatman Protran, Dassel, Germany) and incubated with anti-PAR antibody as described above.

Oxy-2D-TAU Western blot to analyze histone carbonylation

The difference in the development of the Oxy-2D-TAU Western blots to study carbonylation modification on histones is based on the derivatization with DNPH of histones must be made after first dimension separation, as the charges of histones and histone variants must not be altered. Then, this new procedure differs in the second step. After running the first dimension, slides were cut from mono-dimensional TAU gel and then incubated in a solution of 10 mM of DNPH in a 10% solution of trifluoroacetic acid for 20 min. After three washes with bi-distilled water and 5 minutes of preconditioning with running buffer, derivatized slides for collected histones at 6, 24 and 120 hours of cell culture, were loaded in 15% SDS-PAGE and fixed with stacking gel as described in the previous classical 2D-TAU gel. After second dimension electrophoresis, we performed the transference onto a nitrocellulose membrane at 120 V for 1h. Then, the Oxy-2D-TAU membrane was blocked with BSA 5% in PBS (0.1% Tween) and incubated with anti-DNPH antibody, as described by the manufacturer of the Oxy-blot kit described (OxyBlot Protein Oxidation Detection kit, Millipore Inc, Billerica, MA. USA).

Analysis of proteasome activity

40 µg of soluble protein (nuclear or cellular) extracts were incubated with the Promega Proteasome-Glo Assay Reagent (Promega Bioscience, Madison, WI) for 10 minutes. The chymotrypsin-like proteasome activity was detected as the relative light unit (RLU) generated from the cleaved substrate in the reagent using a luciferase coupled-reaction. Luminescence generated from each reaction condition was detected with a Wallac 1420 VICTOR luminometer until saturation of the reaction. Activity was obtained as the slope of the linear increase of luminescence before reaching saturation and normalized with 19S (Rpn2) subunit content from total and nuclear protein extracts measured by Western blot (anti-Rpn2/S1, Biomol Research Labs; Exeter, UK).

Statistical analysis

For the statistical analysis of the results obtained from proteasome activity assay, cellular GSH levels and DNA synthesis by BrdU incorporation, the mean was taken as the measurement of the main tendency, while standard deviation was taken as the dispersion measurement for three independent experiments. Quantification of western blots and coomassie stained gels to normalize histone content was performed using ImageGauge V4.0 and ImageJ V1.41o (Wayne Rasband, NIH, USA).

A one way analysis of variance was used to establish the difference between 6, 24, 48 and 120h cultured fibroblasts. When an interaction effect was found, multiple comparisons using the Student-Newman-Keuls method post hoc test was performed using the GraphPad PRISM V5.0 (GraphPadSoftware, Inc). The alpha level for statistical significance was set at $p < 0.05$.

Results

NIH3T3 cell proliferation

The NIH3T3 fibroblasts began proliferating 24 h after plating, and continued for the following 24 h (total time of culture 48 h). However, at 72 and 120 h cell proliferation decreased. Thus, fibroblasts reached the cellular confluence after 72h in culture (**Fig. 1A**). We studied the growing curve profile of fibroblasts by counting the cells directly and by using BrdU incorporation to the DNA. Maximal DNA synthesis was observed at 24 h after seeding but it continued actively for another 24 h. Seventy two or more hours after seeding, DNA synthesis was low (**Fig. 1B**).

As we described previously [13, 32], maximal rate of cell proliferation at 24 h coincided with a peak of GSH levels, confirming that during cell proliferation there is an increase of cellular glutathione (**Fig. 1C**).

Histone carbonylation increases during DNA synthesis

We measured histone carbonylation during cell proliferation by derivatizing of carbonyl groups formed in the histones using 2,4-dinitrophenylhydrazine.

Histone extracts were subjected to mono-dimensional WB (**Fig. 2A**) showing that histone carbonylation increased at 24 h (**Fig. 2B**), just when most cells underwent DNA synthesis (**Fig. 1B**). Histone carbonylation decreased considerably 48 h after seeding. At 72 and 120 h (at confluence) the carbonylation of histones further decreased (**Fig. 2A and 2B**).

In addition, the results shown in **Fig. 2** indicate that the most susceptible histones to carbonylation were the histone linker H1 and the histone dimers H3.

To confirm our results obtained by mono-dimensional WB and to clarify which histones may be modified by carbonylation, we developed a new methodology called Oxy-2D-TAU Western blot (see Methods). **Fig. 3** shows that histone linker H1, its variant H1^o and H3.1 (and probably H3.2/H3.3) dimers were highly carbonylated 24 h after seeding.

This carbonylation did not take place when cells ceased dividing. Furthermore, H2A and H2B were not carbonylated.

Histone poly(ADP-ribosyl)ation does not correlate with histone carbonylation

We wondered whether differences in the proportion of the histone carbonylation correlated to poly(ADP-ribosyl)ation, since it was reported that *in vivo* carbonylation is the result of an accumulation of ADP-ribose inside the nucleus [18].

Our results further indicate that most poly(ADP-ribosyl)ated histones were histone H1, H3 dimers, and histone H2B variant (**Fig. 4A**). Histone H4 was not poly(ADP-ribosyl)ated (**Fig. 4A**). We observed that histone poly(ADP-ribosyl)ation was maximal at 24h compared to confluence times of 72 and 120 h of cell culture (**Fig. 4B**). At this time, in which histone poly(ADP-ribosyl)ation was maximal, cells were in an active phase of DNA synthesis (**Fig. 1B**). Then, poly(ADP-ribosyl)ation decreased gradually at 72 h, and 120 h, although this post-translational modification continued high at these time points for H1 and H2B (**Fig. 4A**).

Then, we focused our study on the different histone variants using 2D-TAU Western blot. According to mono-dimensional Western blot results (**Fig. 4**), the highest intensity signals for histone poly(ADP-ribosyl)ation were at 24 h after plating. Results show (**Fig. 5**) that, most modified histones were histone H1, its variant H1⁰, histone H2B and interestingly, histone H3 variants H3.2 and H3.3 in their monomer and dimer forms, but not histone variant H3.1

H2AX phosphorylation and histone carbonylation are not related

Protein carbonylation also occurs as a consequence of oxidative stress [19]. We, thus, tested if it could be related to DNA double strand breaks using histone H2AX phosphorylation, because this post-translational modification marks DNA damage foci [33-37].

We found the H2AX phosphorylation (at residue S139) followed a completely different pattern than histone carbonylation, increasing steadily from 6 h up to 120 h after plating (**Fig. 6A and 6B**), showing the highest levels at cell confluence.

Interestingly, our results indicate that, at least under our experimental conditions, histone carbonylation does not correlate with the increase of DNA damage marked by phosphorylation of H2AX.

Changes in total and nuclear proteasome activity during cell proliferation

To investigate the activation of the proteasome system, we isolated and prepared lysates of nuclei of 3T3 cells and total cell lysates at different times of cell culture. We

observed a clear increase of cellular proteasome activity when cells were synthesizing DNA at 24h compared to confluence time of 120h (**Fig. 7A**). Interestingly, nuclear proteasome activity was increased at 48 h (**Fig. 7B**), just when histone carbonylation signal decreased (**Fig. 2**).

Histone carbonylation is a mechanism by which histones may be recycled by the nuclear proteasome.

To analyze if activation of nuclear proteasome may be involved in removing the excess of histones present in the nucleus after the active phase of DNA synthesis, we studied the carbonylation of histones after inhibition of the proteasome with MG132 at 1 μ M. At this dose, MG132 was not toxic for the cells and the cell viability was \approx 95% after 4 hours in incubation (data not shown). An effective nuclear proteasome inhibition was obtained (**Fig. 8A**). The results showed that histone carbonylation was maximal 24 h (**Fig. 8B**). When the proteasome was inhibited with MG132 24h after seeding, we observed increased levels of carbonylated histones. Interestingly, the results obtained 48 h afterwards showed that carbonylated histones accumulated after nuclear proteasome inhibition (**Fig. 8B**), just when they were removed under control conditions. These results confirmed that activation of the nuclear proteasome activity at this time was involved in histone detoxification.

Histone carbonylation is affected by histone poly(ADP-ribosyl)ation inhibition

Work by Ulrich *et al.* [20] showed that oxidized histones were effectively degraded by nuclear proteasome after its poly(ADP-ribosyl)ation mediated by PARP. In addition,

Wondrak *et al.* described that accumulation of ADP-ribose in the nucleus is a potent histone H1 carbonylating agent [18]. Thus, to decipher if histones poly(ADP-ribosyl)ation is the reason for their carbonylation at proliferating times, we performed experiments with 3-ABA, a strong and specific inhibitor of PARP [20]. The cell viability was $\approx 97\%$ after 24 and 48 hours of incubation with 3-ABA 5 mM (data not shown). After analyzing poly(ADP-ribosyl)ated histones, we observed that this post-translational modification was reduced after 3-ABA treatment, mainly for H1 variants and H2B (**Fig. 9A**). Then we evaluated the content of carbonylated histones after 3-ABA incubation. Interestingly, **Fig. 9B** shows that histone carbonylation was barely affected at 24 h. However, histone carbonylation was increased at 48 h when PARPs were inhibited by 3-ABA.

Discussion

Redox sensing mechanisms play important roles in the nucleus and glutathione appears to be an essential molecule in the control of cell proliferation [16, 17, 35].

Epigenetic signals are sensitive to cellular environment [36]. It is generally considered that acetylation/deacetylation of histone tails play a crucial role in modulating gene expression by masking and unmasking positive charges of Lys residues that in turn influence the interaction of histones with DNA [37]. Similar to this modification, which is enzymatically reversible, carbonylation occurs mostly in Lys and Arg residues nonenzymatically; or by reaction with aldehydes produced as a result of lipid peroxidation or sugar oxidation [38-40]. Furthermore, the existence of peptidyl lysil oxidases (LOX) within the nucleus has been described [41] which interestingly, could modify histone H1 *in vitro* [42].

1.-High histone carbonylation when cells are proliferating

Our results indicated that when most cells were in an active phase of DNA synthesis (**Fig. 1A and 1B**) after 24h of cell culture and high GSH levels were present inside the cells (**Fig. 1C**), histone carbonylation was high (**Fig. 2**), mainly for histone H1, H1⁰, and H3 dimers. We confirmed the high susceptibility of linker histone H1 to be carbonylated, as was previously described [18,19].

Using a new method called Oxy-2D-TAU Western blot, we studied the carbonylated histone variants (**Fig. 3**). Our results demonstrated that most carbonylated histones were histone linker H1, its variant H1⁰, and the histone dimers for histone H3.1, H3.2, and H3.3 (**Fig. 3**). Interestingly, Oxy-2D-TAU Western blot demonstrated that histone H3 variants can be carbonylated *in vivo*, contrarily to the results obtained by Sharma *et al.* in which histone H3 was only carbonylated in *in vitro* experiments [19].

Carbonylation masks positive charges of basic amino acids (Lys and Arg residues) changing the chromatin compaction. Thus, histone H3 variants may have been more exposed during the DNA replication process. Another possibility is that higher carbonylation of histones during proliferation may serve as a reactive oxygen species scavenger, as was proposed for young rats in the studies developed by Sharma *et al.* [19]. These authors observed that contrary to the general view, histone proteins were more carbonylated in young than in old rats [19]. In accordance with these reports, we propose that histone carbonylation may act as a shield and thus scavenge the reactive oxygen species with the specific aim of protecting the DNA. This function was previously reported, by Enright *et al.*, since nucleosomal histones might protect DNA from iron-mediated damage [43].

Carbonylation was described to be a consequence of poly(ADP-ribosyl)ation [18]. The accumulation of ADP-ribose within the nuclei could induce a glycation reaction that

eventually produces carbonylation [18]. Our results showed that histone poly(ADP-ribosyl)ation was higher at 24 h than at other culture time points (**Fig. 4**) just when PARPs are described to act after recognition of single strand breaks (SSBs) [44]. Histone carbonylation was increased at 24 h and it occurred at the same time as poly(ADP-ribosyl)ation. However, the pattern for histone carbonylation (**Fig. 2**) does not coincide with poly(ADP-ribosyl)ation (**Fig. 4**). These differences were found in particular histones. In that way, H1, H3 variants, H3 dimers and H2B were poly(ADP-ribosyl)ated. But, only histone H1 and H3 dimers were carbonylated (see **Fig. 2A** and **Fig. 4A**). Histone poly(ADP)ribosylation decreased at cell confluence times (**Fig. 4**), while histone carbonylation was low at all cell culture times except at 24 h (**Fig. 2**). The results for poly(ADP-ribosyl)ation agree with previous results reported by Tanuma *et al.* The authors observed that poly(ADP-ribosyl)ation occurred at all stages of cell cycle [44].

Thus, our results suggested that carbonylation and poly(ADP-ribosyl)ation was related only during the DNA synthesis phase, since the maximal peak for carbonylation and poly(ADP-ribosyl)ation was observed at 24h, for histone H1 and H3 dimers.

2.- Oxy-2D-TAU Western blot, a new tool to study carbonylation in histones

Using our new Oxy-2D-TAU Western blot and comparing the results with those obtained with the 2D-TAU Western blots, a high poly(ADP-ribosyl)ation in H1, H1⁰, H2B, histone variants H3.2/H3.3, was found. However, carbonylation was observed in H1, H1⁰, and H3 dimers. Furthermore, we suggest that there is a relation between carbonylation (**Fig. 3**) and poly(ADP-ribosyl)ation (**Fig. 5**) for the histone linker H1, its variant H1⁰, and the dimers of H3.2/H3.3 variants but not for the dimer of H3.1 or the histone H2B. Thus, our results point out that poly(ADP-ribosyl)ation could induce

histone carbonylation in the particular case of histone H1, its variant H1⁰ and the histone dimers for H3.2/H3.3, but not for the histone H2B, in which poly(ADP-ribosyl)ation did not correlate with histone carbonylation, or in the particular case of histone H3.1, in which we observed carbonylation in its dimer form, but not poly(ADP-ribosyl)ation. These differences observed between the reactivity of histone H3 variants suggested the unique behavior of the histone H3 variants, reinforcing the “histone barcode hypothesis” proposed by Hake and Allis, in which histone H3 variants possess distinct functions [7].

3.- γ -H2AX and histone carbonylation are not associated during cell proliferation

Carbonylation could be produced as consequence of DNA damage. However, we should emphasize that this model of histone carbonylation is not a model of cell aging but of cell proliferation. As expected, γ -H2AX levels were low during the DNA synthesis and were increased when the DNA synthesis was low (**Fig. 6**). It is known that cell cycle is a redox cycle [45, 46] and we previously reported high GSH content into the cell nucleus when most cells were in the S phase [13], decreasing to the lowest levels when most fibroblasts were in the G0/G1 phase of the cell cycle. Then, the low cellular GSH levels (**Fig. 1C**) would make the DNA more susceptible to DNA damage, which could explain the increase of γ -H2AX mark (**Fig. 6**). Our experimental model did not show any correlation between histone carbonylation and γ -H2AX levels.

4.-Carbonylation of histones as a mark for its degradation by the proteasome

Cells have developed mechanisms to reduce oxidative damage to DNA during replication [47], and histones and their ability to condense the chromatin appear as an optimal instrument to scavenge reactive oxygen species and protect DNA [43].

However, the expression of histones should be stoichiometrically coupled to DNA replication, presumably because of their toxicity [48]. However, it is known that histones are expressed in higher amounts during S phase [49-51] and higher amounts of histones may result in toxicity for the cells [52, 53]. Thus, we consider that aside of their chemical role as protection of the integrity of DNA, described by Sharma *et al.* [19] and in the present report; the histone carbonylation process is a mark for their degradation or recycling.

Our results indicate that total proteasome activity was higher (**Fig. 7A**) during DNA synthesis (**Fig. 1B**), probably recycling the excess of synthesized histones by ubiquitination-dependent mechanism. Furthermore, nuclear proteasome activity was activated at 48 h (**Fig. 7B**), just when carbonylated histone levels decreased (**Fig. 2**), suggesting that activation of nuclear proteasome may be involved in removing the excess of histones present in the nucleus, thus may be involved in the detoxification of excess of histones.

An elegant work published by Grune's group revealed that the proteolytic activity of the proteasome increased via poly(ADP-ribosyl)ation, and this activation enhanced the selective degradation of oxidatively damaged histones [54], explaining that carbonylation of histones may serve as a mark for histone degradation by the proteasome. Grune's group found that the degradation of poly(ADP-ribosyl)ated histones was poorly efficient in their model [54]. The present results indicates that the conversion of poly(ADP-ribosyl)ation to carbonylation is necessary for an efficient detoxification process of the excess of some histones produced during S phase, that results toxic for cells [55].

In order to demonstrate if activation of nuclear proteasome is involved in removing the excess of histones present in the nucleus after the active phase of DNA synthesis, we reevaluated our data using the chemical inhibitor of the proteasome MG132 at 1 μ M. This dose of MG132 was effective in inhibiting nuclear proteasome (**Fig. 8A**) and our results show that histone carbonylation increased after such inhibition (**Fig. 8B**). Interestingly, when it was inhibited we observed carbonylated histones at 48 h, just when they would have been removed under control conditions (**Fig. 8B**). These results confirm that activation of the nuclear proteasome activity at 48 h is necessary for histone detoxification and further confirm our hypothesis that nuclear proteasome may be involved in the detoxification of an excess of histones produced after the DNA synthesis.

To establish whether histone poly-(ADP-ribosyl)ation is the reason for carbonylation of histones as proposed by Wondrak *et al.* [18], we proceeded to inhibit PARPs with 3-ABA, a strong and specific inhibitor of PARP [20]. After 3-ABA treatment, poly-(ADP-ribosyl)ation levels of histones were decreased (**Fig. 9A**). Then we analyzed the histone carbonylation levels and observed that they were increased at 48 h when PARPs were inhibited by 3-ABA (**Fig. 9B**). This result suggests that carbonylation was not mainly a consequence of poly(ADP-ribosyl)ation as Wondrak proposed [18]. Interestingly, pioneer work by Ulrich *et al.* [20] showed that oxidized histones were degraded by the nuclear proteasome after the poly(ADP-ribosyl)ation of proteasome mediated by PARP-1. Thus, as we deduced from **Fig. 9A** and **9B**, PARP activity may be critical for the correct function of nuclear proteasome by facilitating the degradation of carbonylated histones.

These results agree with those obtained by Grune's group in which nuclear proteasome is activated by PARP-1 [20, 56], and further support the idea that inhibition of PARP up-regulation by 3-ABA may abolish proteasome activity in proliferating cells, as was previously described for proliferating leukemia cells [57, 58]. Although these results do not rule out that carbonylation is a consequence of poly(ADP-ribosyl)ation of histones, the increase of histone carbonylation after 3-ABA treatment further suggests the possibility of the involvement of additional mechanisms for histone carbonylation, and one of these mechanisms could be mediated by lysyl oxidases (LOXs) [42].

Thus, a harmonious and correct interaction between PARPs, histone poly(ADP-ribosyl)ation, histone carbonylation, nuclear proteasome activation and probably LOXs is necessary for the correct histone detoxification process after DNA synthesis.

Conclusion

We propose that histone carbonylation is a post-translational histone modification directly related to cell proliferation and histone degradation (**Fig. 10**). This modification may be linked with poly(ADP-ribosyl)ation, although this is not the main process that can produce histone carbonylation (e.g H3.1 variant). Here we demonstrate that the histone carbonylation process is not linked to γ -H2AX, a mark related to DNA double strand breaks signaling. Our findings show that histone carbonylation is a physiological process produced when cells are in an active phase of cell proliferation (**Fig. 10A**), suggesting that histones may act as a shield against oxidative species in order to protect the integrity of the DNA during DNA synthesis phase. In addition, carbonylation may be a signal related to the detoxification process in order to eliminate the excess of histones in the nucleus by the poly(ADP-ribosyl)ated "activated" nuclear proteasome,

produced during S phase or just when cells decrease the rate of DNA synthesis (**Fig. 10B**). This process may complete the histone detoxification mechanisms inside the nucleus in order to avoid the side effects produced by excess of histones [52-53]. On the other hand, the use of a new methodology “Oxy 2D-TAU Western blot” to identify the carbonylated histone variants will improve the study of the chromatin structure and nuclear detoxification processes.

The physiological implications of histone carbonylation may help us to understand the prominent role of this modification in epigenetics and chromatin remodeling. Finally, our results corroborate the idea that inhibition of PARPs, is a feasible strategy to increase cytotoxicity because it induces the accumulation of carbonylated histones after nuclear proteasome down-regulation.

Acknowledgements

This work was supported by SAF2008-01338 grants from the *Ministerio de Ciencia e Innovación* and financial support from the CIBERER (Biomedical Network Research Center for Rare Diseases) and the ISCIII to FVP. JLG-G thanks the *Conselleria de Sanitat de la Generalitat Valenciana* for the grant which allowed his stay in Hake's laboratory.

Author Disclosure Statement

The authors declare that no competing interests exist.

List of Abbreviations

BrdU: Bromodeoxyuridine

DNPH: 2,4-dinitrophenylhydrazine

GSH: L- γ -glutamyl-L-Cysteinylglycine

LOX: Lysil oxidase-like proteins

PARPs: Poly-(ADP-ribosyl) polymerases

SSB: single strand break

TAU: Triton-acid acetic-urea

References

- [1] Brown, D.T.; Izard, T.; Misteli, T. Mapping the interaction surface of linker histone H1⁰ with the nucleosome of native chromatin in vivo. *Nat. Struct. & Mol. Biol.* 13:250-255; 2006.
- [2] Cheung, P.; Allis, C.D.; Sassone-Corsi, P. Signaling to chromatin through histone modifications. *Cell.* 103:263–271; 2000.
- [3] Khan, A.U.; Krishnamurthy, S. Histone modifications as key regulators of transcription. *Front. Biosci.* 10:866–872; 2005.
- [4] Iniguez-Lluhi, J.A. For a healthy histone code, a little SUMO in the tail keeps the acetyl away. *ACS Chem. Biol.* 1:204–206; 2006.
- [5] Thompson, P.R.; Fast, W. Histone citrullination by protein arginine deiminase: is arginine methylation a green light or a roadblock? *ACS Chem. Biol.* 1:433–441; 2006.
- [6] Nightingale, K.P.; O'Neill, L.P.; Turner, B.M. Histone modifications: signaling receptors and potential elements of a heritable epigenetic code. *Curr. Opin. Genet. Dev.* 16:125–136; 2006.
- [7] Hake, S.B.; Allis, C.D. Histone H3 variants and their potential role in indexing mammalian genomes: The “H3 barcode hypothesis”. *Proc. Natl. Acad. Sci. USA.* 103(17):6428-6435; 2006.
- [8] Nigg, E.A.; Baeuerle, P.A.; Luhrmann, R. Nuclear import-export: in search of signals and mechanisms. *Cell.* 66:15-22; 1991.
- [9] Zhang, Q.; Piston, D.W.; Goodman, R.H. Regulation of corepressor function by nuclear NADH. *Science* 295:1895-1897; 2002.
- [10] Bürkle, A.; Schreiber, V.; Dantzer, F.; Oliver, F.J.; Niedergang, C.; de Murcia, G.; and Ménissier-de Murcia, J. Biological significance of poly(ADP-ribosylation) reactions: Molecular and genetic approaches. In *From DNA damage and stress signalling to cell death: Poly ADP-ribosylation reactions* (eds. G. de Murcia and S. Shall). Oxford University Press, New York; 2000: 80–124.
- [11] Meister, A.; Anderson, M.E. Glutathione. *Annu Rev Biochem* 52:711-760; 1983.
- [12] Schafer, F.Q.; Buettner, G.R. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.* 30:1191-212; 2001.

- [13] Markovic, J.; Borrás, C.; Ortega, A.; Sastre, J.; Vina, J.; Pallardo, F.V. Glutathione is recruited into the nucleus in early phases of cell proliferation. *J. Biol. Chem.* 282:20416-20424; 2007.
- [14] Conour, J.E.; Graham, W.V.; Gaskins, H.R. A combined in vitro/bioinformatic investigation of redox regulatory mechanisms governing cell cycle progression. *Physiol. Genomics.* 18(2):196-205; 2004.
- [15] Markovic, J.; Mora, N.J.; Broseta, A.M.; Gimeno, A., de la Concepción, N.; Viña, J., Pallardó, F.V. The depletion of nuclear glutathione impairs cell proliferation in 3T3 fibroblasts. *PLoS ONE* 4, e6413; 2009.
- [16] Markovic, J.; García-Giménez, J.L.; Gimeno, A.; Viña, J.; Pallardó F.V. Role of glutathione in cell nucleus. *Free Radic. Res.* 44:721-733; 2010.
- [17] Pallardó, F.V.; Markovic, J.; García-Giménez, J.L.; Viña, J. Role of nuclear glutathione as a key regulator of cell proliferation. *J. Mol. Asp. Med.* 30:77-85; 2009.
- [18] Wondrak, G. T. Histone carbonylation in vivo and in vitro. *Biochem J.* 351:769-777; 2000.
- [19] Sharma, R.; Nakamura, A.; Takahashi, R.; Nakamoto, H.; Goto, S. Carbonyl modification in rat liver histones: Decrease with age and increase by dietary restriction. *Free Radic. Biol. Med.* 40:1179-1184; 2006.
- [20] Ullrich, O.; Reinheckel, T.; Sitte, N.; Hass, R.; Grune, T.; Davies, K.J. Poly-ADP-ribose polymerase activates nuclear proteasome to degrade oxidatively damaged histones. *Proc. Natl. Acad. Sci. USA* **96**: 6223-6228; 1999
- [21] Belenky P., Bogan, K.L., Brenner, C. NAD⁺ metabolism in health and disease. *Trends Biochem. Sci.* 32:12-19; 2007.
- [22] Ziegler, M. New functions of a long-known molecule. Emerging roles of NAD in cellular signaling. *Eur. J. Biochem.*, 267:1550-1564; 2000.
- [23] Berger, F.; Ramírez-Hernández, M.H.; Ziegler, M. The new life of a centenarian: signaling functions of NAD(P)⁺. *Trends Biochem. Sci.* 29:111-118; 2004.
- [24] Corda, D.; Di Girolamo M. Functional aspects of protein mono(ADP-ribosylation). *EMBO J.* 22:1953-1958; 2003.
- [25] Okazaki, I.J.; Moss, J. Characterization of glycosylphosphatidylinositol-anchored, secreted, and intracellular vertebrate mono(ADP-ribosyl)transferases. *Annual Review of Nutrition.* 19: 485–509; 1999.
- [26] Diefenbach, J., Bürkle, A. Introduction to poly(ADP-ribose) metabolism. *Cell. Mol. Life Sci.* 62 (7-8):721–30; 2005.

- [27] Pellny TK, Locato V, Vivancos PD, Markovic J, De Gara L, Pallardó FV, Foyer CH. Pyridine nucleotide cycling and control of intracellular redox state in relation to poly-(ADP-ribose) polymerase activity and nuclear localization of glutathione during exponential growth of Arabidopsis cells in culture. *Mol Plant* 2(3): 442-456, 2009.
- [28] Bouliskas T. Poly(ADP-ribosylated) histones in chromatin replication. *J. Biol. Chem.* 265 (24):14636-47, 1990.
- [29] Vichai,V.; Kirtikara, K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat. Prot.* 1:1112-1116; 2006.
- [30] Schechter, D.; Dormann, H.L.; Allis, C.D.; Hake, S.B. Extraction, purification and analysis of histones. *Nat. Prot.* 2(6):1445-1457; 2007.
- [31] Shacter, E.; Williams, J. A.; Lim, M.; Levine, R. L. Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay. *Free Radic. Biol. Med.* 17:429-437; 1994.
- [32] Borrás, C.; Esteve, J.M.; Viña, J.R.; Sastre, J.; Viña, J.; Pallardó, F.V. Glutathione Regulates Telomerase Activity in 3T3 Fibroblasts. *J. Biol. Chem.* 279:34332-34335; 2004.
- [33] Rouse J.; Jackson, S.P. Interfaces between the detection, signaling and repair of DNA damage. *Science.* 297:547-551; 2002.
- [34] Wang, C.; Jurk, D.; Maddick, M.; Nelson, G.; Martin Ruiz, C.; von Zglinicki T. DNA damage response and cellular senescence in tissues of aging mice. *Aging Cell.* 8:311-323; 2009.
- [35] Jones, D. P. Redox sensing: orthogonal control in cell cycle and apoptosis signalling. *J. Intern. Med.* 268:432-448; 2010.
- [36] Bonasio R., Tu, S., Reinberg, D. Molecular Signals of Epigenetic states. *Science.* 330:612-616; 2010.
- [37] Jenuwein, T.; Allis, C.D. Translating the histone code, *Science.* 293:1074–1080; 2001.
- [38] Stadtman, E. R. Protein oxidation and aging. *Science.* 257:1220– 1224; 1992.
- [39] Stadtman, E. R.; Levine, R. L. Protein oxidation. *Ann. N.Y. Acad. Sci.* 899:191– 208; 2000.
- [40] Requena, J. R.; Chao, C-C.; Levine, R.L.; Stadtman, E.R. Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal catalyzed oxidation of proteins. *Proc. Natl. Acad. Sci. USA.* 98:69-74; 2001.

- [41] Li, W.; Nellaiappan, K.; Strassmaier, T.; Graham, L.; Thomas, K.M.; Kagan, H.B. Localization and activity of lysyl oxidase within nuclei of fibrogenic cells. *Proc. Natl. Acad. Sci. USA.* 94:12817-12822; 1997.
- [42] Kagan, H.M; Li W. Lysyl oxidase: properties, specificity and biological roles inside and outside of the cell. *J. Cell. Biochem.* 88:660-672; 2003.
- [43] Enright, H. U.; Miller, W. J.; Hebbel, R. P. Nucleosomal histone protein protects DNA from iron-mediated damage. *Nucleic Acids Res.* 20: 3341– 3346; 1992.
- [44] Tanuma S-I., Enomoto T., Yamada M-A. Changes in the level of poly-ADP-ribosylation during cell cycle. *Exp. Cell Res.* 117(2):421-430; 1978.
- [45] Menon, S.G.; Goswami P.C. A redox cycle within the cell cycle: ring in the old with the new. *Oncogene.* 26(8):1101-1109; 2007.
- [46] Menon, S. G., Redox regulation of the G1 to S phase transition in the mouse embryo fibroblast cell cycle. *Cancer Res.* 63:2109-2117; 2003.
- [47] Klevecz, R.R.; Bolen, J.; Forrest, G.; Murray, D.B. A genomewide oscillation in transcription gates DNA replication and cell cycle. *Proc Natl Acad Sci U S A.* 101(5):1200-5; 2004.
- [48] Jaeger, S.; Barends, S.; Giegé, R.; Eriani, G.; Martin, F. Expression of metazoan replication-dependent histone genes. *Biochimie.* 87:827-834; 2005.
- [49] Muskhelishvili, L.; Latendresse, J.R.; Kodell, R.L.; Henderson, E.B. Evaluation of cell proliferation in Rat Tissues with BrdU, PCNA, Ki-67(MIB-5) immunohistochemistry and In situ Hybridization for histone mRNA. *J. Histochem. Cytochem.* 51(12):1681-1688; 2003.
- [50] Sive, H.L.; Heintz, N.; Roeder, R.G. Regulation of human histone gene expression during the HeLa cell cycle requires protein synthesis. *Mol. Cell. Biol.* 4(12):2723-2734; 1984.
- [51] Zhu, Z.; Edwards, R.J.; Boobis, A.R. Increased expression of histone proteins during estrogen-mediated cell proliferation. *Environ. Health Perspect.* 117(6):928-934; 2009.
- [52] Xu, J.; Zhang, X.; Pelayo, R.; Monestier, M.; Ammollo, C. T.; Semeraro, F.; Taylor, F.B.; Esmon N.L.; Lupu, F.; Esmon, C. T. Extracellular histones are major mediators of death in sepsis. *Nat. Med.* 15:1318-1321; 2009.
- [53] Chaput C., Zychlinsky A. Sepsis: the dark side of histones. *Nat. Med.* 15(11): 1245-1246; 2009.

- [54] Catalgol, B.; Wendt, B.; Grimm, S.; Breusing, N.; Özer, N.K.; Grune, T. Chromatin repair after oxidative stress: Role of PARP-mediated proteasome activation. *Free Radic. Biol. Med.* 48:673-680; 2010.
- [55] Singh, R. K.; Kabbaj, M-H. M.; Paik, J., Gunjan, A. Histone levels are regulated by phosphorylation and ubiquitylation-dependent proteolysis. *Nat. Cell. Biol.* 11(8): 925-934; 2009.
- [56] Bakondi, E.; Catalgol, B.; Bak, I.; Jung, T.; Bozaykut, P.; Bayramicli, M.; Kartal Ozer, N.; Grune, T. Age-related loss of stress-induced nuclear proteasome activation is due to low PARP-1 activity. *Free Radic. Biol. Med.* **50**: 86-92; 2011
- [57] Ciftci, Ö.; Ullrich, O.; Schmidt, A.; Diestel A.; Hass, R. Regulation of the nuclear proteasome activity in myelomonocytic human leukemia cells after adriamycin treatment. *Blood* **97**:2830- 2838; 2001
- [58] Ullrich, O.; Grune, T. Proteasomal degradation of oxidatively damaged endogenous histones in K562 human leukemic cells. *Free Radic. Biol Med.* **31**:887-893; 2001
- [59] Nakamura, A.; Kawakami, K.; Kametani, F.; Nakamoto, H.; Goto, S. Biological Significance of protein modifications in aging and calorie restriction. *Ann. N. Y. Acad Sci.* 197:33-39; 2010.

Figures legends

Figure 1. Profile of cell proliferation and cellular GSH levels in NIH3T3 fibroblasts. **A)** Figure shows the growth curve by counting of the cells directly at each time of cell culture. **B)** Measurement of DNA synthesis determined by BrdU incorporation assay. **C)** Cellular GSH levels measured by the GSH transferase enzymatic assay. Results are shown as mean (\pm SD) of three different experiments by triplicate. ANOVA analysis and a posthoc Student-Newman-Keuls method were performed to analyze statistical differences between groups ($n = 3$, $*p < 0.05$; $**p < 0.005$).

Figure 2. Changes in histone carbonylation levels during NIH3T3 cell proliferation. **A)** Representative pattern of histone carbonylation obtained from NIH3T3 fibroblasts during cell growth at different times of culture. Extracted histones were derivatized using DNPH. After electrophoresis in a 15% SDS-PAGE and WB using an anti-DNP primary antibody, carbonylated histone patterns were compared at the different times of cell culture (left). Coomassie blue stained gel of extracted histones corresponding to the same gel after the transference of the proteins to the nitrocellulose membrane (right). **B)** Quantification of carbonylated histones during cell growth at different times of culture (means \pm SD, $n = 3$, $*p < 0.05$).

Figure 3. 2D TAU-SDS-PAGE gels for carbonylated histones obtained from NIH3T3 fibroblasts. Representative oxy-2D-TAU pattern of histone carbonylation

obtained from NIH3T3 fibroblasts during three selected times of cell cycle (left); [6 h: cells start to proliferate; 24 h: maximum of DNA synthesis, and 120 h: cell confluence]. Extracted histones were separated using a TAU gel. After the first dimension separation, histones were derivatized using DNPH (10 mM) acid solution. Then, the lanes for each condition were cut and the strips were deposited in a 15% SDS-PAGE for the second dimension. After electrophoresis, the gels were transferred onto nitrocellulose membrane. Membranes were blocked and then incubated with anti-DNP primary antibody. Carbonylated histone patterns were compared at the different times of cell culture. Coomassie blue stained gel of extracted histones (right) is the same gel after the transference of the proteins to the nitrocellulose membrane.

Figure 4. Changes in poly(ADP-ribosyl)ated histones within the nucleus during 3T3 cell proliferation. **A)** Representative mono-dimensional pattern of histone poly(ADP-ribosyl)ated obtained from NIH3T3 fibroblasts during cell proliferation (left). Coomassie blue stained gel of extracted histones (right). **B)** Quantification of poly(ADP-ribosyl)ated histones during cell growth at different times of culture (means \pm SD, n = 3, * p < 0.05).

Figure 5. 2D TAU-SDS-PAGE gels for poly(ADP-ribosyl)ated histones obtained from NIH3T3 fibroblasts. Representative 2D-TAU-SDS-PAGE pattern of histone poly(ADP-ribosyl)ated obtained from NIH3T3 fibroblasts at three selected times of cell cycle (left); [6 h: cells start to proliferate; 24 h: maximum of DNA synthesis, 120 h: cell confluence]. Extracted histones were separated using a TAU gel. After the first dimension separation, the lanes for each condition were cut and the strips were

deposited in a 15% SDS-PAGE for the second dimension. After electrophoresis, gels were transferred onto a nitrocellulose membrane. Then, the membranes were blocked and incubated with anti-poly(ADP-ribosyl)ation antibody. Poly(ADP-ribosyl)ation histone patterns were compared at the different times of cell culture. Coomassie blue stained gel of extracted histones (right) is the same gel after the transference of the proteins to the nitrocellulose membrane.

Figure 6. Levels of gamma-H2AX during cell proliferation of NIH3T3 cells. A)

Changes in the levels of phospho-H2A.X during NIH3T3 cell proliferation. Coomassie gel representing the distribution of histones in a 15% SDS-PAGE obtained from NIH3T3 fibroblasts during cell proliferation (top image). Phospho-H2AX levels at different times of cell proliferation (middle image). Levels of H2AX variant during cell proliferation (bottom image). **B)** Quantification of gamma-H2AX during cell growth at different times of culture (means \pm SD, $n = 2$, $*p < 0.05$).

Figure 7. Total and nuclear proteasome activity during cell proliferation of NIH3T3 cells.

Normalized proteasome activity in **A)** cellular extracts and **B)** nuclear extracts obtained from NIH3T3 fibroblasts at different times of cell culture. Results are shown as mean (\pm SD) of three different experiments by triplicate. ANOVA analysis and a posthoc Student-Newman-Keuls method were performed to analyze statistical differences between groups ($n = 3$, $*p < 0.05$).

Figure 8. Deciphering histone carbonylation metabolism after proteasome inhibition using MG132 1 μ M.

A) Nuclear proteasome activity analysis at 24 and 48 h of NIH3T3 cell culture after 4 h of MG132 1 μ M treatment as described in *Materials and Methods*. Results are shown as means \pm SD of three different experiments by triplicate. ANOVA analysis and a posthoc Student-Newman-Keuls method were performed to analyze statistical differences between groups (n =3, *p<0.05). **B)** Changes in histone carbonylation at 24 and 48 h of cell culture after treatment with MG132 1 μ M, Oxy blot using anti-DNP primary antibody (left). Coomassie gel of extracted histones after proteasome inhibition (right). Control cells were incubated with the vehicle DMSO.

Figure 9. Deciphering histone carbonylation metabolism after PARP inhibition by 3-ABA 5 mM.

A) Representative western blot of poly(ADP-ribosyl)ated proteins from total extracts of NIH3T3 cells at 24 and 48 h of cell culture treated with 3-ABA 5 mM. Western blot of poly(ADP-ribosyl)ated histones at 24 and 48 h of cell culture after treatment with 3-ABA 5 mM (left). Coomassie gel of extracted histones after PARP inhibition (right). **B)** Analysis of histone carbonylation in NIH3T3 cells at 24 and 48 h of cell culture after treatment with 3-ABA 5 mM (left). Coomassie gel of extracted histones after PARP inhibition (right). Control cells were incubated with the vehicle DMSO.

Figure 10. Proposed mechanism for histone carbonylation production and detoxification. **A)** Production of Histone carbonylation during S-phase. The cell supplies to the newly synthesized DNA strands an excess of histones that ensure the

correct package by the chromatin. Cells may activate some mechanisms to mark the excess of histones to be degraded by the proteasome. The excess of the newly synthesized histones may be recycled by the proteasome after their synthesis. Additionally, poly-(ADP-ribosyl)ated histones could be susceptible of carbonylation after glycation/glicoxidation reaction as proposed by Wondrak *et al.* [18]. Also, lysil oxidases-like proteins may produce the carbonylation of Lys residues of histones as was suggested Nakamura A *et al.* [59]. **B)** During the late S-phase, the synthesis of histones decreases and the nuclear proteasome is highly activated, probably by poly-(ADP-ribosyl)ation, as it has been suggested by Ullrich *et al.* [20]. This activation reduces the level of carbonylated histones and allows the correct conformation of the chromatin in their two differentiated forms (euchromatin and heterochromatin).

PARPs: Poly-(ADP-ribose) polymerases. LOX: Lysil oxidases-like proteins. RI-histones: replication independent histones.